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Agronomic performance and genetic characterization of sugarcane transformed for resistance to sugarcane yellow leaf virus

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ABSTRACT

Sugarcane yellow leaf virus (SCYLV, a *Poleovirus* of the *Luteoviridae* family) is already widespread in Florida, and resistance in the Canal Point (CP) sugarcane population is limited. Genetic transformation of sugarcane for disease resistance holds promise but tissue culture and transformation processes produce undesirable agronomic characteristics necessitating thorough field evaluation. A 3-year sugarcane (a complex hybrid of *Saccharum* species) field study was conducted in Belle Glade, FL with the following objectives: (1) thoroughly evaluate the agronomic performance of two transgenic lines transformed for SCYLV resistance (6-1, 6-2) compared with parental cultivar control CP 92-1666, (2) determine level of SCYLV resistance in the transgenic lines, and (3) characterize genetic differences in the transgenic lines compared with CP 92-1666 using simple sequence repeat (SSR) genotyping. Sugarcane yields of CP 92-1666 were superior to both transgenic lines, as well as tissue culture (C-1) and nptII marker gene (20-1) controls, in the plant cane, first ratoon and second ratoon crops. CP 92-1666 recorded an average of 6.5–8.7 tons sucrose ha^{-1} yr^{-1} more than genotypes subjected to tissue culture and biolistic transformation. However, SCYLV infection rates in transgenic lines were only 0–5%, compared with 98% in CP 92-1666. Kanamycin field assays indicated that selectable marker gene nptII was stably expressed in all co-transformed lines. SSR genotyping showed 35 additional fragments to be present and 25 existing fragments absent among 6-1, 6-2, C-1 and 20-1 compared with CP 92-1666. Although all clones had unique genotypes, the four regenerated clones showed a greater genetic distance from the donor clone CP 92-1666 (mean GD 0.4) than to one another (mean GD 0.03). This study reports the first successful gene transfer of SCYLV resistance in sugarcane and the first report of variations in microsatellite repeat number associated with regeneration from embryogenic callus. Our results highlight the potential of genetic transformation methods to incorporate desirable traits into sugarcane, combined with the necessity of thorough agronomic evaluation of transgenic genotypes. Transgenic lines 6-1 and 6-2 are being used as parents in crosses designed to combine SCYLV resistance from these genotypes with agronomic characteristics of high-yielding materials.

1. Introduction

Sugarcane yellow leaf virus symptoms in sugarcane are characterized by a yellowing of the abaxial surface in the upper leaves. In severe cases growth is stunted leading to a fan-like appearance in the plant. These symptoms are caused by a luteovirus (Scaglusi and Lockhart, 2000; Moonan et al., 2000; Smith et al., 2000), which has been associated with a phytoplasma in some regions (Aljanabi et al., 2001). There are several virus strains which are associated with SCYLV and these may differ in infection capacity and virulence (Ahmad et al., 2006, 2007). The virus is transmitted by aphid vectors (Schenck and Lehrer, 2000; Lehrer et al., 2007), and may also be spread through infected vegetative planting material.

Sugarcane yellow leaf was first described less than 20 years ago in Hawaii (Schenck, 1990), but despite its relatively recent discovery has been reported in more than 30 countries (Lockhart and Cronje, 2000). Sugarcane yellow leaf virus symptoms mimic abiotic stresses which made field identification difficult; however, recently published disease surveys indicate that SCYLV has the potential to spread quite rapidly. Incidence of SCYLV infection has been reported in 89% of grower fields in Florida (Comstock et al.,

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1999), in 90% of cultivars surveyed in Ecuador, Guatemala and Honduras (Comstock et al., 2002), in 98% of stalks surveyed in a susceptible cultivar in Reunion (Rassaby et al., 2004), in 73% of cultivars surveyed in Colombia (Victoria et al., 2005), and in 62% of stalks surveyed in the central valley of Costa Rica (Moreira et al., 2006).

There are few published studies on SCYLV effects on sugarcane growth and yield. Physiological changes in SCYLV infected plants include a reduction in leaf area and a decrease in leaf chlorophyll and N content (Izaguirre-Mayoral et al., 2002). Rassaby et al. (2003) reported a 19–37% yield reduction in the first ratoon crop in two susceptible cultivars in Reunion, but yields of cultivar R570 were not affected by SCYLV.

The Canal Point (CP) sugarcane clones occupy more than 90% of total sugarcane area in Florida and generate more than $2 billion in total economic activity. Yield trial data indicate a 4–7% yield loss in cultivars infected with SCYLV (Flynn et al., 2005), thus the spread of SCYLV is a major concern. Traditional breeding techniques to incorporate SCYLV resistance are likely to be lengthy since the degree of SCYLV resistance in the CP sugarcane population appears to be limited. A survey of SCYLV incidence in the CP program indicated that 67% of Stage IV clones (the final field testing stage) and 98% of grower collection clones were infected with the virus (Comstock et al., 1999).

Meristem tip culture of virus-free plants (Fitch et al., 2001) and meristem tissue culture of infected varieties (Parmessur et al., 2002) have reduced SCYLV and phytoplasma pathogens in regenerated plants. However the benefits of meristem tissue culture techniques appear to be transitory in the field under Florida conditions (Comstock and Miller, 2005).

Molecular breeding techniques using microprojectile or *Agrobacterium* mediated gene transfer have been used to incorporate herbicide (Gallo-Meagher and Irvine, 1996; Falco et al., 2000; Leibbrandt and Snyman, 2003), disease (McQualter et al., 2004; Gilbert et al., 2005), and pest (Arencibia et al., 1999; Li-Xing et al., 2006) resistance into sugarcane. Rangel et al. (2005) recently reported successful incorporation of SCYLV resistance into Colombian cultivar CC84-75 through microprojectile gene transfer. While these techniques have the potential to improve the efficiency of sugarcane crop improvement (Lakshmanan et al., 2005), variability in agronomic traits of transformed clones due to somaclonal variation caused by tissue culture and transformation procedures (Arencibia et al., 1999; Carmona et al., 2005; Gilbert et al., 2005; Vickers et al., 2005) necessitate thorough field evaluation. To our knowledge there are no published reports of agronomic evaluation or field disease resistance of sugarcane genetically modified for resistance to SCYLV.

Embryogenic regeneration is known to cause genetic variability in plants, and most reports of variability have involved the analysis of Random Amplified Polymorphic DNA (RAPD) markers. In sugarcane, RAPD markers were used successfully to identify genetic variations following regeneration of embryogenic callus (Taylor et al., 1995) or indeed meristem cultures (Zucchi et al., 2002). Whereas, no genetic variability resulting from the regeneration of sugarcane embryogenic callus was detected by Chowdhury and Vasil (1993) using the analysis of restriction fragment length polymorphisms (RFLPs). The results of RAPD analyses are well known to be difficult to reproduce (MacPherson et al., 1993; Mcunier and Grimont, 1993) making their application in genome analysis limited. A more robust marker system would appear to be the analysis of simple sequence repeats (SSRs) which involves the detection of variations in repeat number at specific loci within the genome. The analysis of SSRs has been applied widely in genome analysis because SSRs are highly polymorphic, easy to analyze, and reproducible (Li et al., 2002; Ellegren, 2004).

The objectives of this study were to (1) evaluate the agronomic characteristics of two transgenic sugarcane genotypes bioengineered for resistance to SCYLV compared with the non-transformed parent clone CP 92-1666, (2) determine field expression of co-transformed SCYLV resistance and selectable marker genes, and (3) identify any genetic differences between transformed clones and parent controls using SSR genotyping.

2. Materials and methods

2.1. Plasmids

Plasmids Ubi-Km and pFM396 were provided by E. Mirkov at the Texas A&M Agricultural Experiment Station, Weslaco, TX. Plasmid pMBP39-22 (Huang et al., 1997) was provided by L. Owens at the Beltsville Agricultural Research Center, USDA, Beltsville, MD. Plasmid pFM396 contains an untranslatable SCYLV coat protein DNA fragment in the antisense orientation and under control of the maize ubiquitin promoter, first exon and first intron, followed by the nopaline synthase terminator. The MB39 gene in pMBP39-22 was amplified by polymerase chain reaction (PCR) using primers CCF2 (5′-TTGAAGATCTCGAGCCATGGGGAAGAAGAGCCACA-3′) AND CCR2 (5′-TTGACTCGAGATCTTATCCTAGCGCTTTGGCTTGC-3′) introducing Xho I restriction sites at both ends of the fragment. The MB39 gene encodes for modified Cecropin B which is an antimicrobial peptide. The MB39 gene and the ScYLV coat protein genes should act independently. The following program was run: initial denaturation for 3 min at 95 °C, followed by 40 cycles of 45 s at 95 °C, 1 min at 52 °C, and 1 min at 72 °C. The final extension step was 8 min at 72 °C. All reactions had a final volume of 20 μL and contained Tris buffer (1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris, pH 8.3), 0.2 mM of each DNTP, 10 pM of each primer, 1 unit of *Taq* polymerase and 5 ng of sample. The MB39 fragment was then restricted with Xho I and ligated into the Sal I site of pFM396 yielding pZY-CSA. Plasmid Ubi-Km contained the nptII gene under control of the maize ubiquitan promoter and was used to select calli for resistance to kanamycin.

2.2. Bombardment and regeneration of transgenic plants

Embryogenic callus was established from young leaf spindle tissues and maintained on MS medium (Murashige and Skoog, 1962) with 0.3% Gellrite and 3 mg/L 2,4-D (MS3 medium). Callus was co-bombarded with pZY-CSA and Ubi-Km-coated tungsten microprojectiles by procedures described by Franks and Birch (1991), except a particle inflow gun constructed and operated as described by Gray et al. (1994) was used. Following bombardment, the calli were put onto MS3 medium for 1 week, and then transferred to MS medium with 1 mg/L 2,4-D and 50 mg/L geneticin for 2 weeks. Plantlets were regenerated on MS medium without 2,4-D containing 65 mg/L geneticin at 28 C under alternating 12 h light and 12 h dark and then transferred to soil. The presence of the MB39 and anti-sense SCYLV coat protein genes in putatively transformed plants was determined by PCR amplification of a 571 bp fragment of the MB39 and anti-sense SCYLV coat protein genes using the CCF1 (5′-TCCGGCCATCAGCC-GAAATGGAAAGT-3′) and FM385F (5′-TGCTAACCGTCGTTGACT-GACTC-3′) primer pair.

2.3. Experimental design

Following transformation and selection, transgenic plants and controls were initially planted in the field in unreplicated plots at

the USDA-ARS Sugarcane Field Station in Canal Point, FL on February 13, 2002. Five genotypes were included in this study: (1) CP 92-1666, non-transformed parental control, (2) C1, non-transgenic line regenerated from CP 92-1666 tissue culture, (3) 20-1, transgenic line with the nptII selectable marker gene only, (4) 6-1, transgenic line with both the nptII selectable marker gene and putative SCYLV resistance gene, and (5) 6-2, transgenic line with both the nptII selectable marker gene and putative SCYLV resistance gene. These genotypes were chosen to allow comparison of transgenic to tissue culture and parental controls. All transgenic lines produced (2) were included in this study. Cultivar CP 92-1666 was released in 1999 with an average sucrose yield 12% greater than commercial control CP 70-1133 (Glaz et al., 2001). While the sucrose yield of parent cultivar CP 92-1666 was acceptable for commercial release, in this study CP 92-1666 was used primarily to introduce the SCYLV resistance gene into commercial germplasm with the goal of using the bioengineered lines thus generated as parents to improve sucrose yield in future generations of crossing.

On February 12, 2003 seed cane for genotypes 2–5 was cut at the USDA-ARS Sugarcane Field Station. Genotype CP 92-1666 seed cane was obtained separately from a commercial trial at Duda Farms. On February 14, 2003 the experiment was planted at the University of Florida, Everglades Research and Education Center (EREC; 26°39′N, 80°38′W) in Belle Glade, FL on a Lauderhill muck (euic, hyperthermic, Lithic Haplosaprist) soil. The experiment was planted in a randomized complete block design with 4 replications, except that two plots of CP 92-1666 controls were included in each replicate. Each plot was a single row 3-m long, with a 1.5-m spacing between rows, and a 3-m bare alley break between adjacent plots.

2.4. Sugarcane yield traits

Yield trait measurements were performed in the plant cane (PC), first ratoon (1R) and second ratoon (2R) crops. Plant population (stalks m^{-2}) was determined in August of 2003 (PC), 2004 (1R) and 2005 (2R). Five stalks were harvested at random to calculate cane production in February 2004 (PC), 2005 (1R), and 2006 (2R). Plant fresh weights were used to determine individual stalk weight (kg $stalk^{-1}$), and tons of cane per hectare (TCH, tons ha^{-1}) were calculated as the product of stalk number and stalk weight. To determine sucrose concentration (KST, kg sucrose ton^{-1}), the five-stalk harvest samples were ground in a three-roller mill and the juice analyzed for Brix and pol. Brix, which is a measure of percent soluble solids, was measured using a refractometer (model RFM91, Bellingham and Stanley, Ltd., Lawrenceville, GA) which automatically corrected for temperature. Pol, which is a measure of the polarization of light passing through the sugar solution, was measured using a saccharimeter (model AP115-589, Rudolph Research Analytical, Hackettstown, NJ). Sucrose concentration was determined according to the theoretical recoverable sugar (TRS) method (Glaz et al., 2002). Sucrose yield (TSH, tons sucrose ha^{-1}) was calculated as the product of TCH and KST (divided by 1000 to convert kilogram sucrose to metric tons). All sugarcane in each plot was harvested mechanically in April of 2003 (PC), 2004 (1R) and 2005 (2R) and the resultant regrowth measured as described above for the following ratoon crop. Following second ratoon harvest, all transgenic sugarcane was destroyed in the field according to USDA-APHIS protocols.

Analyses of variance for sugarcane yield traits were performed using Proc Mixed in SAS® (Littell et al., 2002). Separate analyses were run for data pooled across crop cycles and data pooled across genotypes, which were the fixed effects in the model, whereas replications were random effects. Least squares estimates in SAS were used to determine probabilities levels associated with differences among crop cycle or genotype means.

Fig. 1. Leaf of sugarcane genotype CP 92-1666 showing leaf chlorosis due to kanamycin susceptibility 1 week following spray of 1 mL kanamycin solution at the rate of 3.0 g L^{-1} into leaf whorl. Kanamycin resistant plants (nptII selectable marker gene expressed) did not develop chlorosis symptoms.

2.5. SCYLV disease assays

Leaf samples for genotypes 1–5 were collected at EREC in May and June 2004. SCYLV infection was determined by assaying for the presence of the virus using a tissue blot immunoassay using antibodies specific for the virus. The top fully emerged leaf was removed and the basal portion of the leaf blade was cut with a sharp razor-blade scalpel and the freshly cut midrib was firmly pressed on a nitrocellulose membrane leaving a clear impression of the midrib. The membrane was serologically developed using SCYLV specific antibodies developed by B.E. Lockhart, University of Minnesota according to Schenck et al. (1997) except that Fast Blue was used as the enzyme substrate. SCYLV positive samples were identified by the blue strained areas where the phloem cells left impressions.

2.6. Selectable marker expression test

Expression of the nptII selectable marker gene was tested in the field using a kanamycin antibiotic assay (Falco et al., 2000). A 3.0 g L^{-1} solution of kanamycin monosulfate (Agri-Bio, Miami, FL) was combined with 2 mL L^{-1} surfactant (Silwet L-77, Helena Chemical Co., Collierville, TN) and deionized water in a 1 L spray bottle. A stream of approximately 1 mL was sprayed into the leaf whorl of 3 randomly selected stalks in each plot on March 29, 2004. Each sprayed stalk was marked with flagging tape for subsequent observation. On April 5, 2004 each stalk was scored as either kanamycin susceptible (leaf chlorosis symptoms, Fig. 1) or resistant (no leaf chlorosis), with kanamycin resistance indicating expression of the nptII marker gene.

2.7. SSR genotyping

DNA was isolated from 600 mg of inner whorl tissue taken from the growing point of three separate stools of the transformed materials (6-1, 6-2) and the transformed and untransformed checks (20-1 and C-1 respectively) as well as from the donor clone (CP 92-1666) according to methods described in Glynn et al. (2008). Genetic fingerprints for all five genotypes were obtained by SSR genotyping using twelve primer pairs (mSSCIR14, SMC17CG,

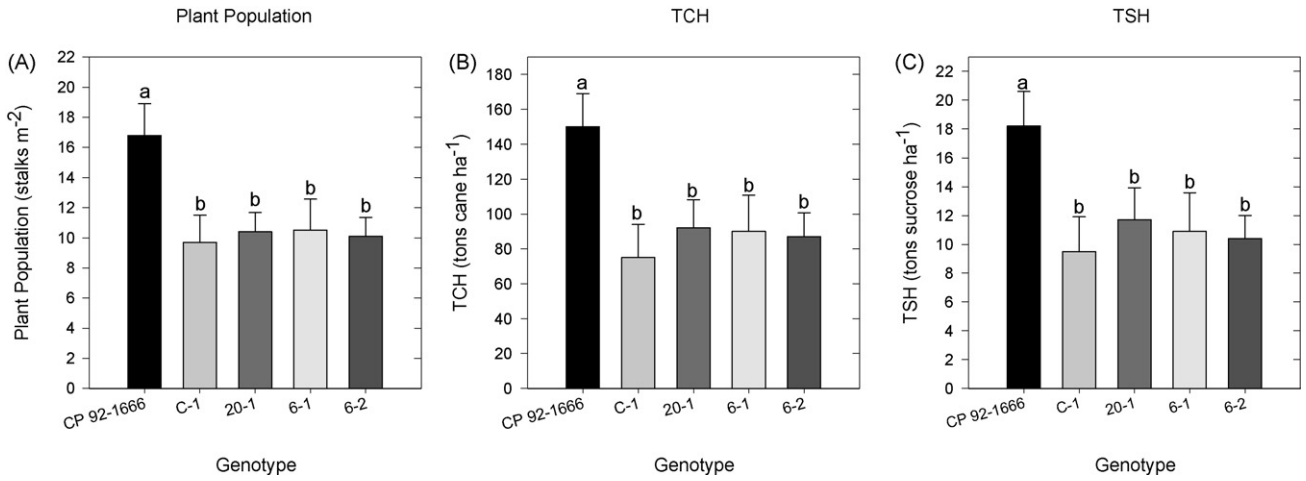

Fig. 2. Effect of genotype on sugarcane yield traits: (A) stalk number, (B) cane yield (TCH), and (C) sucrose yield (TSH). Bars with different letters are significantly different ($P = 0.05$).

mSSCIR53, mSSCIR54, mSSCIR70, SMC179SA, SMC221MS, SMC222CG, SMC334BS, SMC336BS, SMC1493CL, SMC1572CL) developed through the International Consortium of Sugarcane Biotechnology (Cordeiro et al., 2003). PCR amplification and fragment analysis was performed according to methods described in Edmé et al. (2006). A binary matrix for presence and absence of amplified fragments among the five genotypes was generated. For comparison, these data together with data generated from the analysis of the same 12 SSR markers in six commercial sugarcane cultivars was used to produce genetic distance indices and a phenetic tree with Treecon Version 1.3b[1] (Van de Peer and DeWachter, 1994). Distance estimations were performed using default settings.

3. Results

3.1. Sugarcane yield traits

Sugarcane genotype had a significant effect on sugarcane plant population, TCH and TSH, but not on stalk weight or KST (Table 1). Crop cycle had a significant effect on sugarcane plant population, TCH and TSH. Sucrose concentration (KST) was not significantly affected by crop cycle. In general sugarcane sucrose yield differences were caused by differences in biomass yield rather than sucrose content. The interaction of crop cycle x genotype was not significant for any sugarcane yield trait (Table 1), thus data were pooled across crops or genotypes for data analysis.

Commercial cultivar CP 92-1666 recorded clearly superior sugarcane yield traits compared with other genotypes which were subjected to tissue culture and/or transgenesis in this study (data pooled across crop cycles). CP 92-1666 plant population was 6.3–7.1 stalks m^{-2} (Fig. 2A), TCH 56–75 tons cane ha^{-1} (Fig. 2B), and TSH 6.5–8.7 tons sucrose ha^{-1} (Fig. 2C) greater than other genotypes. There were no significant differences in sugarcane yield traits between genotypes C-1, 20-1, 6-1 or 6-2.

Sugarcane plant population (Fig. 3A) and stalk weights (Fig. 3B) recorded different trends among crops (data pooled across genotypes), with plant population increasing by 42% and stalk weight decreasing by 56% from plant cane to second ratoon. However, the overall trend was towards decreasing biomass yield (Fig. 3C) and sucrose yield (Fig. 3D) in ratoon crops. Average TCH declined from 130 tons cane ha^{-1} in plant cane to 82 tons cane ha^{-1} in second ratoon, while TSH also declined from 15.8 tons sucrose ha^{-1} in plant cane to 9.8 tons sucrose ha^{-1} in second ratoon.

3.2. SCYLV disease resistance

SCYLV infection rates recorded by laboratory assays were much greater for parental control CP 92-1666 than sugarcane clones transformed for resistance to SCYLV (Table 2). Leaves of CP 92-1666 were infected by SCYLV in 98% of the assays, but only 5% of the leaves from genotype 6-1 and 0% from genotype 6-2 were infected with SCYLV. Genotype 20-1, which possessed the selectable marker gene but not the SCYLV resistance gene, also exhibited low infection rates, whereas genotype C-1, which had been subjected to tissue culture without transgenesis, exhibited intermediate infection rates.

3.3. Selectable marker expression

The expression of antibiotic resistance via the selectable marker gene nptII was confirmed by the kanamycin field assay. The three genotypes transformed with nptII all recorded 100% resistance to kanamycin in the field (Table 2), whereas the commercial and tissue culture controls without the nptII gene developed leaf chlorosis symptoms (Fig. 1) on all plants sprayed with kanamycin.

3.4. SSR genotyping

Different SSR fingerprints were obtained for each of the five clones tested (6-1, 6-2, C-1, 20-1 and CP 92-1666). The fingerprints were identical for each of the three replicate DNA samples. The 12 SSR primers produced 107 fragments, of these, 41 and 66

Table 1
Analysis of variance F ratios and level of significance for sugarcane plant population, stalk weight, sucrose concentration (KST), biomass yield (TCH) and sucrose yield (TSH).

Fixed effects	Plant population	Stalk weight	KST	TCH	TSH
Crop (C)	15.7**,†	164***	4.9	12.9**	13.9**
Genotype (G)	23.2***	1.1	1.0	17.5***	16.3***
C × G	1.2	0.9	1.6	0.8	1.3

† $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

[1] Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by USDA.

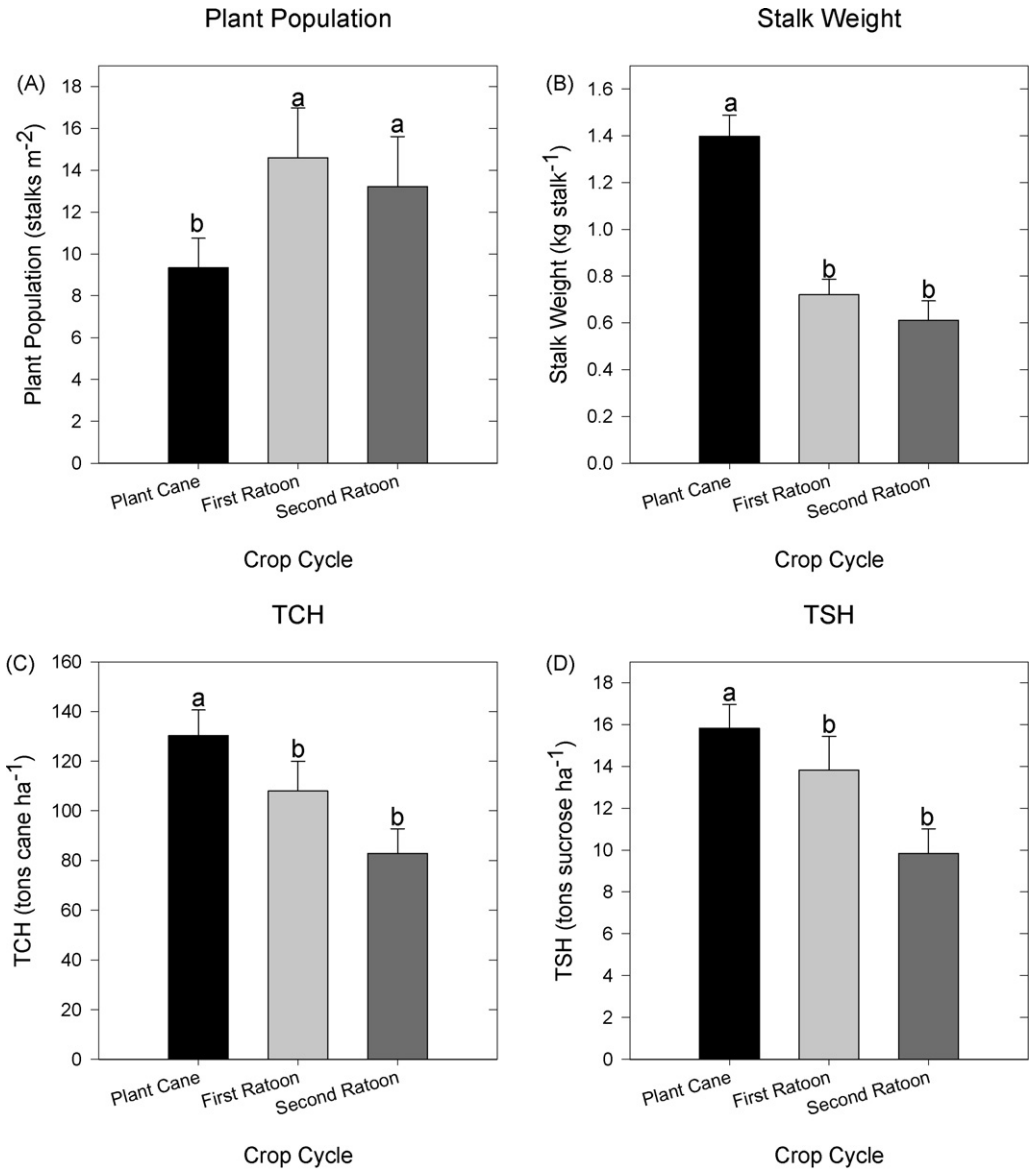

Fig. 3. Effect of crop cycle on sugarcane yield traits: (A) stalk number, (B) stalk weight, (C) cane yield (TCH), (D) sucrose yield (TSH). Bars with different letters are significantly different ($P = 0.05$).

fragments were mono and polymorphic respectively. Thirty-five fragments were not present in CP 92-1666 but were present in at least one of the other four genotypes, 31 of which were present in all four clones. Twenty-five fragments were present in CP 92-1666 yet absent from any of the other four clones. The fragment profiles obtained for the test clones 6-1, 6-2, C-1 and 20-1 differed from the donor clone CP 92-1666 for all 12 of the SSR's examined. The number of fragments amplified from these four genotypes

Table 2
Genotype sugarcane yellow leaf virus (SCYLV) field infection determined by lab assay, and expression of the selectable marker gene (nptII) determined by kanamycin field assay.

Genotype[a]	SCYLV assays	SCYLV infected[b]	Kanamycin assays	Selectable marker expression[c]
	No. leaves	%Leaves	No. plants	%Plants
CP 92-1666	77	98	24	0
C-1	84	24	12	0
20-1	84	4	12	100
6-1	85	5	12	100
6-2	83	0	12	100

[a] Genotypes included CP 92-1666 (commercial control), C-1 (tissue culture only), 20-1 (tissue culture and nptII gene), 6-1 and 6-2 (tissue culture, nptII gene and SCYLV resistance gene).
[b] SCYLV field infection determined by tissue blot immunoassay.
[c] Plants not expressing nptII gene exhibited leaf chlorosis in the field following spraying with kanamycin solution at 3.0 g L^{-1} (see Fig. 1).

Table 3
Summary of SSR data for twelve primer pairs used to produce genetic fingerprints of CP 92-1666 and four genotypes derived from tissue culture following co-bombardment with pZY-CSA and Ubi-Km (6-1 and 6-2), bombardment with Ubi-Km only (20-1) and no bombardment (C-1).

Primer	Total loci	Band in CP 92-1666	Number of bands additional (+) to or absent (–) from CP 92-1666 in test clones							
			6-1		6-2		20-1		C-1	
			+	–	+	–	+	–	+	–
mSSCIR14	8	7	1	3	1	3	1	3	1	3
SMC17CG	5	5	0	3	0	3	0	3	0	3
mSSCIR53	5	3	2	0	2	0	2	0	2	0
mSSCIR54	15	8	7	4	7	4	7	4	7	4
mSSCIR70	9	5	3	3	3	3	4	3	3	3
SMC179SA	7	5	2	1	2	1	2	0	2	0
SMC221MS	12	7	4	1	4	1	5	2	5	2
SMC222CG	3	3	0	1	0	1	0	1	0	1
SMC334BS	8	6	2	4	2	4	2	4	2	4
SMC336BS	14	8	5	3	5	3	5	3	5	3
SMC1493CL	7	5	2	2	2	2	2	2	2	2
SMC1572CL	14	10	3	3	3	3	4	4	4	4

increased for primers mSSCIR53, mSSCIR54, mSSCIR70, SMC179SA, SMC336BS, and SMC221MS, was reduced for primers mSSCIR14, SMC17CG, SMC222CG, SMC334BS and SMC1572CL and remained the same for primer SMC1493CL compared with CP 92-1666 (Table 3). Some variations in the fragments amplified among the four test genotypes were evident (Table 3). Fingerprints from all five samples were different to one another, genetic distance analysis showed them to differ from several of the major sugarcane varieties cultivated in Florida (Fig. 4).

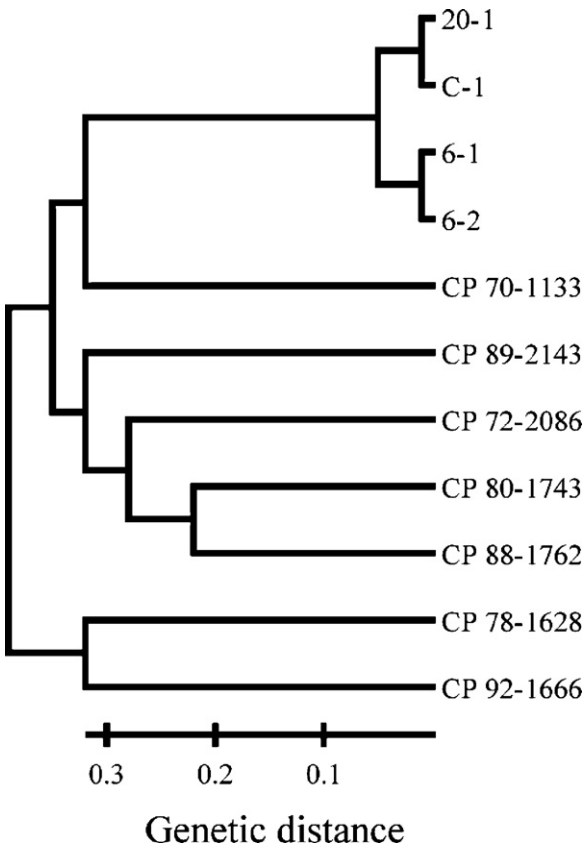

Fig. 4. A phenetic tree based on genetic distance estimates for four genotypes derived from tissue culture following co-bombardment with pZY-CSA and Ubi-Km (6-1 and 6-2), bombardment with Ubi-Km only (20-1) and no bombardment (C-1) together with the donor clone (CP 92-1666) and six other commercial sugarcane cultivars grown in Florida.

4. Discussion

Our results demonstrate a strong dichotomy between agronomic performance and field disease resistance in the transgenic lines generated. The parental control CP 92-1666 recorded clearly superior yields to the transformed lines. Our results concur with Vickers et al. (2005), who also reported unacceptable agronomic performance of sugarcane subjected to tissue culture and biolistic transformation. However, there are other reports (Leibbrandt and Snyman, 2003; Gilbert et al., 2005) of transgenic sugarcane lines with acceptable agronomic performance equivalent to commercial checks. Large numbers of transgenic lines may be needed to select clones with acceptable agronomic performance (Gilbert et al., 2005; Vickers et al., 2005). Sugarcane cultivars respond differently to transformation (Gilbert et al., 2005), and difficulties obtaining viable lines from CP 92-1666 precluded us from generating more than two lines for this study.

SCYLV field infection rates dropped from 98% in the commercial parent CP 92-1666 to 0–5% in transgenic lines in this study. Previous results using artificial inoculation in Colombia (Rangel et al., 2005) also indicate that SCYLV resistance can be successfully incorporated using microprojectile plasmid bombardment techniques. Transgenic lines 6-1 and 6-2 are being used as parents to insert SCYLV resistance into more robust genotypes. Thorough agronomic and SCYLV resistance evaluation of seedlings from crosses involving 6-1 and 6-2 will be necessary to identify agronomically acceptable genotypes. The use of transgenic lines with SCYLV resistance is likely to constitute a quicker option than traditional breeding techniques as SCYLV resistance in the CP sugarcane population is quite limited (Comstock et al., 1999).

The incidence of SCYLV infection was lower in all clones subjected to tissue culture than the parental control. These results agree with previous reports of SCYLV reduction using apical meristem culture (Schenck and Lehrer, 2000; Fitch et al., 2001; Parmessur et al., 2002). The use of tissue culture alone would be a simpler approach than transgenesis due to the restrictions imposed on transgenes. However, sugarcane treated with tissue culture may become reinfected when exposed to SCYLV virus challenge (Schenck and Lehrer, 2000).

The decline in sugarcane yields from the plant cane to second ratoon crop in this study is typical of yield trends in Florida. Only 13% of all Florida sugarcane acreage was in third ratoon or older crops in 2005 (Glaz, 2006).

All sugarcane plants transformed with the nptII gene in 2000 expressed antibiotic resistance in 2004, indicating stable expression of the gene through two cycles of vegetative reproduction. Our

selectable marker screening results thus concur with Falco et al. (2000) and indicate that the kanamycin assay is a simple and useful technique to detect nptII selectable marker gene expression in the field.

The analysis of SSR fragments showed clearly that the four genotypes tested differed from the parental donor clone (CP 92-1666). Variation was associated with callus regeneration and was consistent between samples taken from separate stools indicating that these variations are stable. The degree of variation exhibited caused genetic distance estimates between the donor and test clones to be greater than between the test clones and several other commercial varieties which emphasizes the severity of the induced mutations. However, the physiological characters (bud, auricle and stalk morphology) of the test clones were in general consistent with those reported for CP 92-1666 (Schueneman et al., 2002). Therefore, despite suffering significant reductions in yield and severe alterations in SSR fingerprint patterns the test clones did retain some of the genetic identity of the original clone.

Several of the SSR markers used in this study have been mapped in previous studies. Markers mSSCIR14 and 53 mapped to homology group (HG) I and mSSCIR54 and 70 to HG VI and III respectively in R570 (Rossi et al., 2003) whereas markers SMC336BS and SMC1572CL mapped to HG II, SMC179SA and SMC1493CL to HG II and IV respectively in the Australian cultivar Q165 (Aitken et al., 2005). This suggests that the changes observed are genome wide rather than localized.

Although effects of callus regeneration have been detected in sugarcane and other crops through the analysis of RAPD fragments, to the best of our knowledge, this is the first report of such variations detected through the analysis of SSR's. Alteration in SSR repeat length in nature occurs due to replication slippage (Levinson and Gutman, 1987) as a result of the DNA polymerase pausing (Hile and Eckert, 2004). A small fraction of initial mutations not corrected by the mismatch repair system result in insertion or deletion of repeat units (Strand et al., 1993). The majority of fragments that differed from CP 92-1666 were either present or absent from all of the other four clones. This suggests that the mechanism of mutation induction is relatively consistent and may be dependant upon the donor genotype or regeneration conditions employed. Determination of the basis of these alterations and whether they occur as a result of alterations in DNA polymerase replication or to the mismatch repair system may lead to strategies that reduce the genetic and resulting phenotypic variability associated with the regeneration of sugarcane from embryogenic callus. Analysis of these genotypes using markers such as AFLP may help elucidate whether the variations observed here are associated specifically with SSR or other loci.

5. Conclusion

This study reports the first field evaluation of sugarcane transformed for SCYLV resistance. Transgenic clones 6-1 and 6-2 exhibited low yield potential combined with high levels of resistance to SCYLV. SSR genotyping revealed significant genetic differences between transformed lines and their parents which may explain their poor agronomic performance. Our results highlight the potential of genetic transformation methods to incorporate desirable traits into sugarcane, combined with the necessity of thorough field evaluation of transgenic genotypes. While transgenic lines 6-1 and 6-2 were not acceptable for commercial production, they are being used as parents in crosses designed to combine SCYLV resistance from these genotypes with agronomic characteristics of high-yielding germplasm.

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